Supplementary Figures

Sup Figure 1. H3N1 infection causes AHR and inflammation in WT and NKT cell deficient mice.

a-c. 8 wk old BALB/c (**a-b**, n=15 per group) or C57BL/6 (**c**, n=5 per group) mice, treated with influenza A virus (Mem71, H3N1) or control allantoic fluid (mock-infection), were assessed 5, 10 and 15 days post-infection for AHR. (**a,c**) Changes in lung resistance (Penh, enhanced pulse) were measured. ***p<0.001 compared to mock-infected group. (**b**) Cells in BAL fluid were collected and the numbers of macrophage (Mac), neutrophil (Neu), eosinophil (Eos) and lymphocyte (Lym) were analyzed 10 days after the virus challenge (right panel). ***p<0.001 compared to mock infected group.

d. Representative lung sections stained with H&E from mock or H3N1-infected BALB/c or C57BL/6 mice on day 10.

e-f. 8 wk old Wt or *Cd1d*$^{-/-}$ mice (n=4 per group) treated with H3N1 or control allantoic fluid, were assessed 5 days post-infection for AHR. Changes in lung resistance (**e**) and cells in BAL fluid were analyzed (**f**). Data are representative of three independent experiments.

Sup Figure 2. TLR7 agonist induce production of IL-33 in alveolar macrophages.

AM were infected *in vitro* with the indicated amount of poly I:C or R848 or influenza A (H3N1; M.O.I=5; 2X10^5 cells/well, in 96 well plates) for 96 hrs. Supernatants were analyzed for IL-33 by ELISA (eBiosciences). Data are representative of two independent experiments.

Sup Figure 3. Natural helper cells secrete IL-5.

a. Lung cells were taken from H3N1 or mock infected BALB/c or *Rag2*$^{-/-}$ mice on day 5 and stimulated with or without PMA + ionomycine for 5 hr. The percentage of lung CD45$^+$lin$^-$ST2$^+$ Sca-1$^+$ cells was assessed by FACS. The second row panels show dot plots for lin$^-$ST2$^+$cells in lung leukocytes (CD45$^+$). After gating on the Lin$^-$ST2$^+$ cells, the cells were analyzed for intracellular IL-5 and Sca-1 expression (third row panels).

b. Total RNA from H3N1-infected BALB/c mice lungs was isolated on day 1, 3, 5, 7, or day 14 and analyzed by qRT-PCR for IL-5 mRNA expression.

c. The lungs of H3N1 or mock-infected BALB/c mice (n=3 per group) were taken on days 0, 1, 4, and 7 post-infection. The individual lungs were homogenized in 1ml PBS and then assessed for IL-5 protein by ELISA (eBiosciences). ***p<0.001, compared to mock-infected group. Data are representative of three independent experiments.

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Sup Figure 4. Natural helper cells constitute the major subset of IL-13 secreting cells in the lung after H3N1 infection.

Lung cells were isolated from mock or H3N1-infected mice, and intracellular IL-13 expression was assessed following stimulation with PMA + ionomycin for 5 hr. The percentage of lung IL-13$^+$ cells and lin$^-$ST2$^+$ Sca-1$^+$cells was assessed by FACS. The second row panels show dot plots for IL-13$^+$ cells in the live lung cells (first row panels). After gating on the IL-13$^+$ cells, the cells were analyzed for Lin$^-$ST2$^+$ (blue arrow) and Lin$^-$ST2$^-$ (red arrow, lower panels) cells, and then focused on c-Kit and Sca-1 staining (bottom row panels). Data are representative of three independent experiments.

Sup Figure 5. IL-13 secretion from natural helper cells and alveolar macrophages.

a. Lung cells were taken from H3N1 or mock infected wt mice on day 1 and further stimulated with PMA + ionomycin for 5 hr. The percentage of lung CD45$^+$lin$^-$ ST2$^+$c-Kit$^+$Sca-1$^+$ cells was assessed by FACS. Upper panels show dot plots for Lin$^+$ST2$^+$ and Lin$^-$ST2$^+$cells in lung leukocytes (CD45$^+$). After gating on the Lin$^-$ST2$^+$c-Kit$^+$cells, the cells were analyzed for intracellular IL-13 and Sca-1 expression (lower panels).

b. Lung cells from (**a**) were assessed by FACS for the percentage of lung interstitial macrophage (IM; F4/80$^+$CD11c$^-$), alveolar macrophage (AM; F4/80$^+$CD11c$^+$) and dendritic cells (DC; F4/80$^-$CD11c$^{+)}$ in lung leukocytes (CD45$^+$). Intracellular IL-13 expression and the absolute cell number were further analyzed by gating on these subsets.

c. Alveolar macrophages (AM), bone marrow-derived dendritic cells (BMDC) or a mouse lung epithelial cell line (MLE) were infected with H3N1 (M.O.I=5; upper panel); alveolar macrophages (AM) were infected with H3N1 (M.O.I=5; 0.5; 0.05; lower panel) for 24 hr *in vitro*. Total RNA was extracted from the cells and analyzed by qRT-PCR for IL-13 mRNA expression. *$p<0.05$, ***$p<0.001$, compared to mock-infected group.

d. Lung cells were taken from H3N1 or mock infected BALB/c mice on day 5 and further stimulated with PMA + ionomycin for 5 hr. The percentage of lung T cells (TCRβ^+) among lung leukocytes (CD45$^+$) (upper panels), and intracellular IL-13 and IFN-γ expression in CD4$^+$ and CD8$^+$ T cells (middle and lower panels) were assessed by FACS. Data are representative of three independent experiments.

Sup Figure 6. *Ifn$\gamma^{-/-}$* mice develop robust H3N1-induced AHR.

a. Total RNA from H3N1-infected BALB/c mice lungs was isolated on day 1, 3, 6, 9 or day 15 and analyzed by qRT-PCR for IFN-γ mRNA expression.

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b-e. 8 wk old Wt, *Tbx21*$^{-/-}$ or *Ifnγ*$^{-/-}$ mice (a-b, n=5-6 per group), treated with H3N1 or mock infected with allantoic fluid, were assessed 5 days post-infection for AHR. (**b, d**) Changes in lung resistance (R_L) as a function of methacholine dose are reported. (**c, e**) Cells in BAL fluid were collected and analyzed 5 days after virus challenge. Data are representative of three independent experiments.

Sup Figure 7. Natural helper cells do not produce IL-17 after H3N1 infection.
Lung cells were isolated from mock or H3N1-infected mice, and intracellular IL-17 expression was assessed following by stimulation with PMA + ionomycin for 5 hr. The percentage of CD45$^+$Lin$^-$ST2$^+$ cells was assessed by FACS. After gating on the CD45$^+$Lin$^-$ST2$^+$ cells (top panels), the cells were analyzed for intracellular IL-17 and Sca-1 expression (bottom panels). Data are representative of three independent experiments.

Sup Figure 8. Kinetics of viral clearance in mice.
The lungs of H3N1-infected Wt, *Rag2*$^{-/-}$, *Il13*$^{-/-}$ and *Il1rl1(St2)*$^{-/-}$ mice were taken on the indicated days after infection and assessed for influenza virus by qRT-PCR. The data are presented as relative PFU/lung on a log scale. Data are representative of two independent experiments.

Sup Figure 9. Anti-Thy1.2 (CD90) mAb treatment of *Rag2*$^{-/-}$ mice depletes Natural Helper cells.
a. Lung cells were taken from H3N1 or mock-infected wild-type mice on day 1 and the percentage of lung CD45$^+$Lin$^-$ST2$^+$ Sca-1$^+$ cells was assessed by FACS.
The upper panels show dot plots for Lin$^+$ST2$^+$ and Lin$^-$ST2$^+$ cells in lung leukocytes (CD45$^+$). After gating on the Lin$^-$ST2$^+$c-Kit$^+$cells, the cells were analyzed for Thy1.2 and Sca-1 expression (lower panels). The results indicate that H3N1 infection increases the number of Sca-1$^+$ natural helper cells in the lung. Data are representative of three independent experiments.
b. 8 wk-old *Rag2*$^{-/-}$ mice were treated with three injections of anti-Thy1.2 mAb (clone 30-H12; 0.5 mg, day -3,0 day 3), and were then infected with H3N1 virus on day 0. The lung cells were taken from H3N1 infected wt mice on day 5 and the percentage of lung CD45$^+$Lin$^-$ST2$^+$Sca-1$^+$ cells was assessed by FACS. The lower panels show dot plots for Lin$^+$ST2$^+$ and Lin$^-$ST2$^+$ cells in lung leukocytes (CD45$^+$) after Thy1.2 treatment, and indicate that anti-Th1.2 mAb treatment depleted the Thy1.2$^+$Lin$^-$ST2$^+$ natural helper cells.
c. protocol for adoptive transfer of natural helper cells to *Il13*$^{-/-}$ recipients.

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Sup Figure 10. *In vitro* stimulation of natural helper cells induced IL-5 and IL-13 secretions.

a. Lung natural helper cells (lin^-ST2^+) were isolated from the donors that received IL-33 (1μg, i.n.), and the purity of lin^-ST2^+ cells were assessed by FACS after sorting.

b-c. Lung natural helper cells (lin^-ST2^+) were isolated from the naïve *Rag2-/-* donors (4×10^4 cells/well, 96 well plates) were cultured with 50 ng/ml IL-2, 100 ng/ml IL-33 or IL-2 plus IL-33 for 24hrs to 6 days *in vitro*. PMA + ionomycin stimulation was used as a positive control and no cytokine (IL-2 (-)) was used as negative control. Total RNA was extracted from the cells after 24hrs culture, and analyzed by qRT-PCR for IL-5 and IL-13 mRNA expression. Supernatants from triplicate wells were collected on day 1, day 4 and day 6, and then assessed for IL-5 and IL-13 protein by ELISA.

d. After 4 days of culture, the cells were harvested and analyzed by FACS for intracellular cytokine expression following stimulation with PMA + ionomycin for 5 hr. The percentage of $CD45^+lin^-ST2^+$ cells in the live cell gate was assessed by FACS. After gating on the $CD45^+lin^-ST2^+$ cells (top panels), the cells were analyzed for intracellular IL-5, IL-13, IFN-γ, IL-4 and IL-17, compared with respective isotype control. Data are representative of three independent experiments.

Sup Figure 11. Schematic showing a macrophage-natural helper cell immune axis leading to acute AHR after influenza virus infection.

Influenza A virus infects and activates alveolar macrophages (AM), interstitial macrophages (IM), dendritic cells (DC), or lung epithelial cells, resulting in the secretion of IL-33 within 24hr of virus infection. IL-33 production in turn activates natural helper cells (nuocytes) through ST2 receptors. This interaction leads to IL-13 production in natural helper cells (nuocytes) 5-6 days after infection. The IL-13 then drives mucus secretion by airway epithelial cells and the development of AHR.

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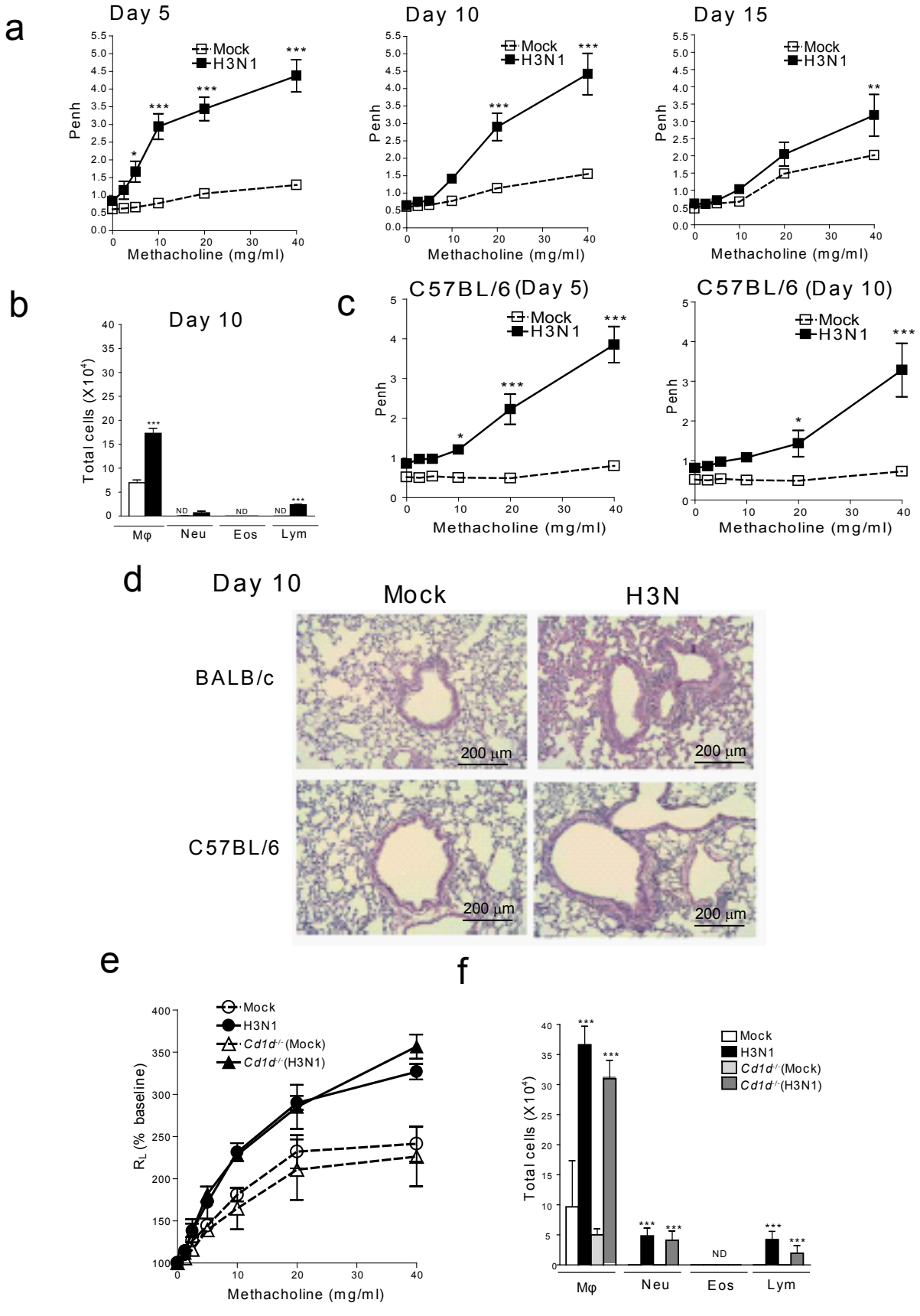
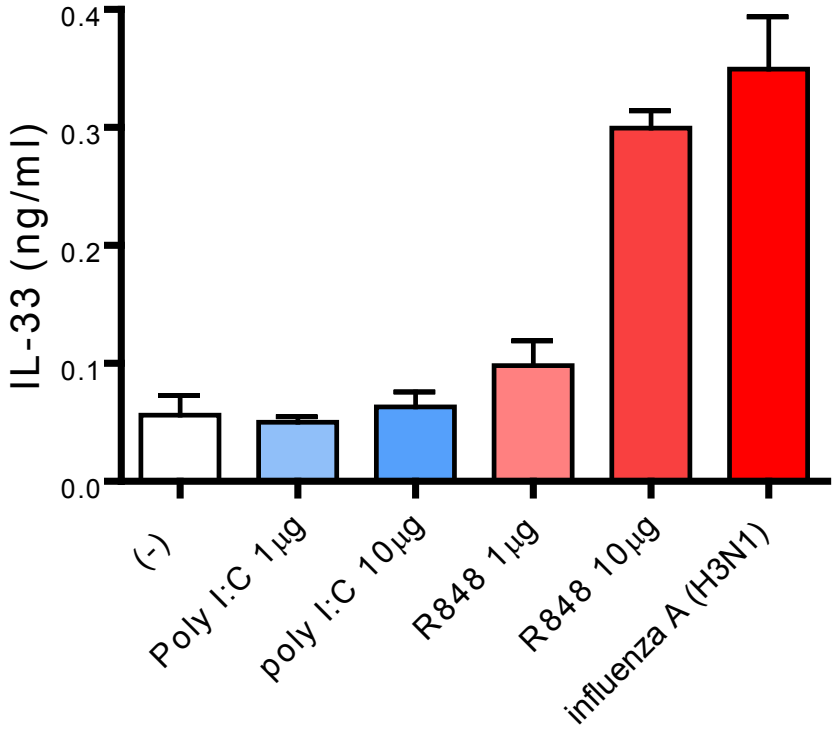
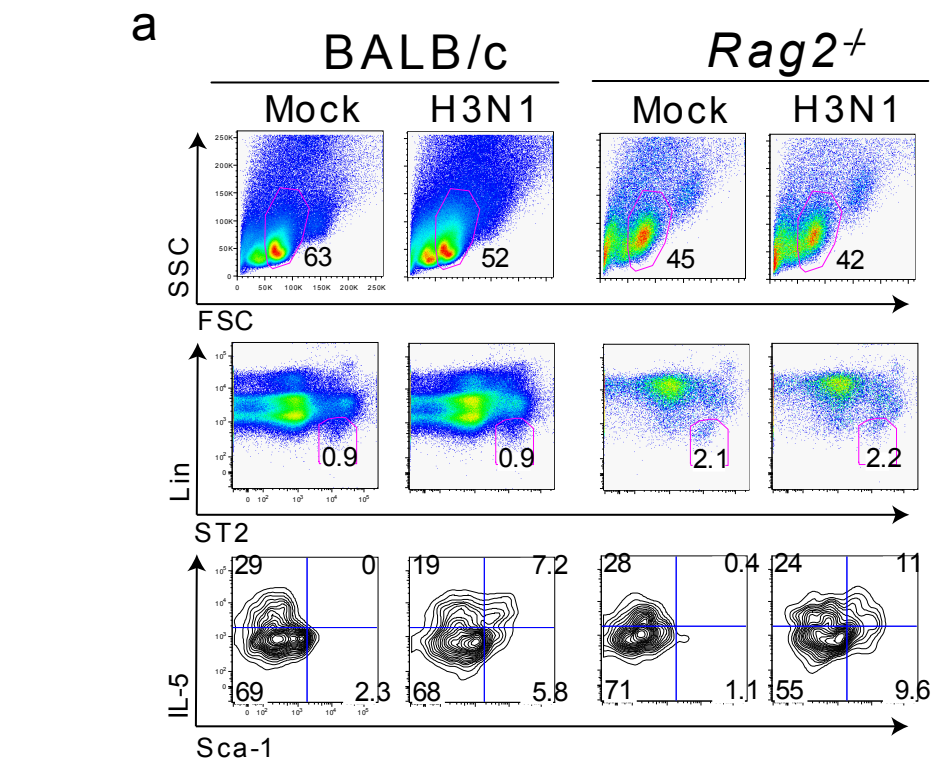
Sup Fig 1

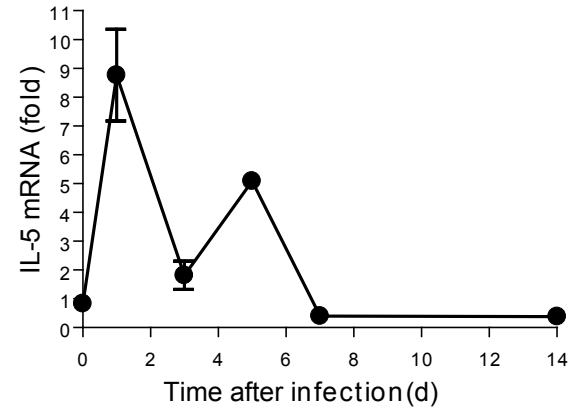

a
Day 5
Day 10
Day 15
Mock
H3N1
Penh
Methacholine (mg/ml)
b
Day 10
Total cells (X10⁴)
Mφ
Neu
Eos
Lym
ND
c
C57BL/6 (Day 5)
C57BL/6 (Day 10)
d
Day 10
Mock
H3N
BALB/c
C57BL/6
200 μm
e
Cd1d-/- (Mock)
Cd1d-/- (H3N1)
RL (% baseline)
f

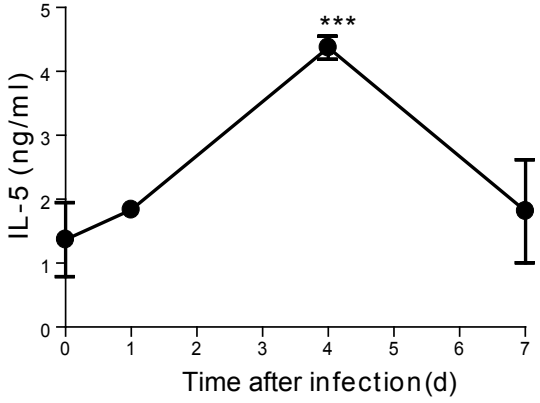
Sup Fig 2


IL-33 (ng/ml)
0.4
0.3
0.2
0.1
0.0
(-)
Poly I:C 1μg
poly I:C 10μg
R848 1μg
R848 10μg
influenza A (H3N1)

Sup Fig 3

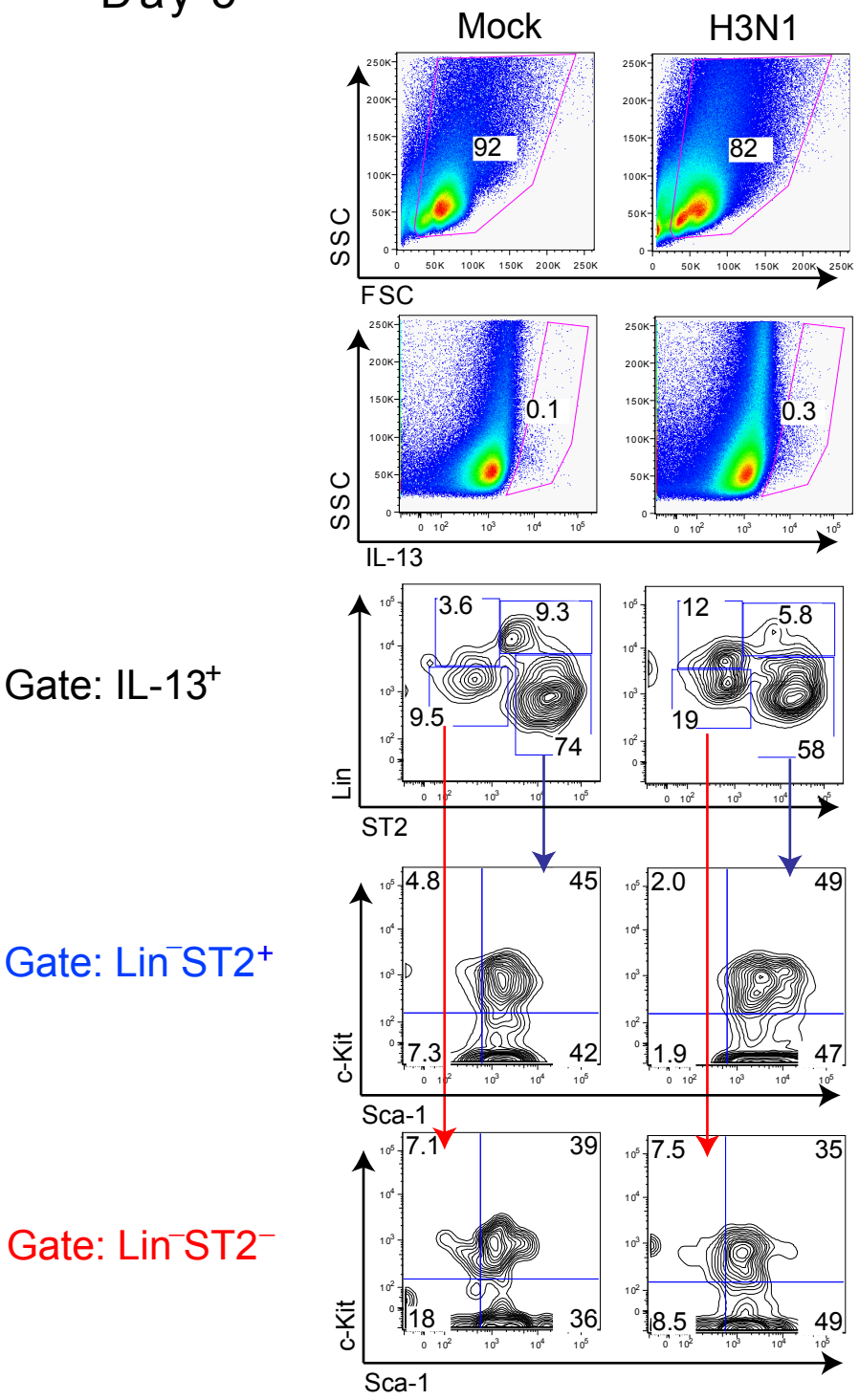

a
BALB/c
Rag2-/-
Mock
H3N1
Mock
H3N1
SSC
FSC
63
52
45
42
Lin
ST2
0.9
0.9
2.1
2.2
IL-5
Sca-1
29
0
69
2.3
19
7.2
68
5.8
28
0.4
71
1.1
24
11
55
9.6


b
IL-5 mRNA (fold)
Time after infection (d)

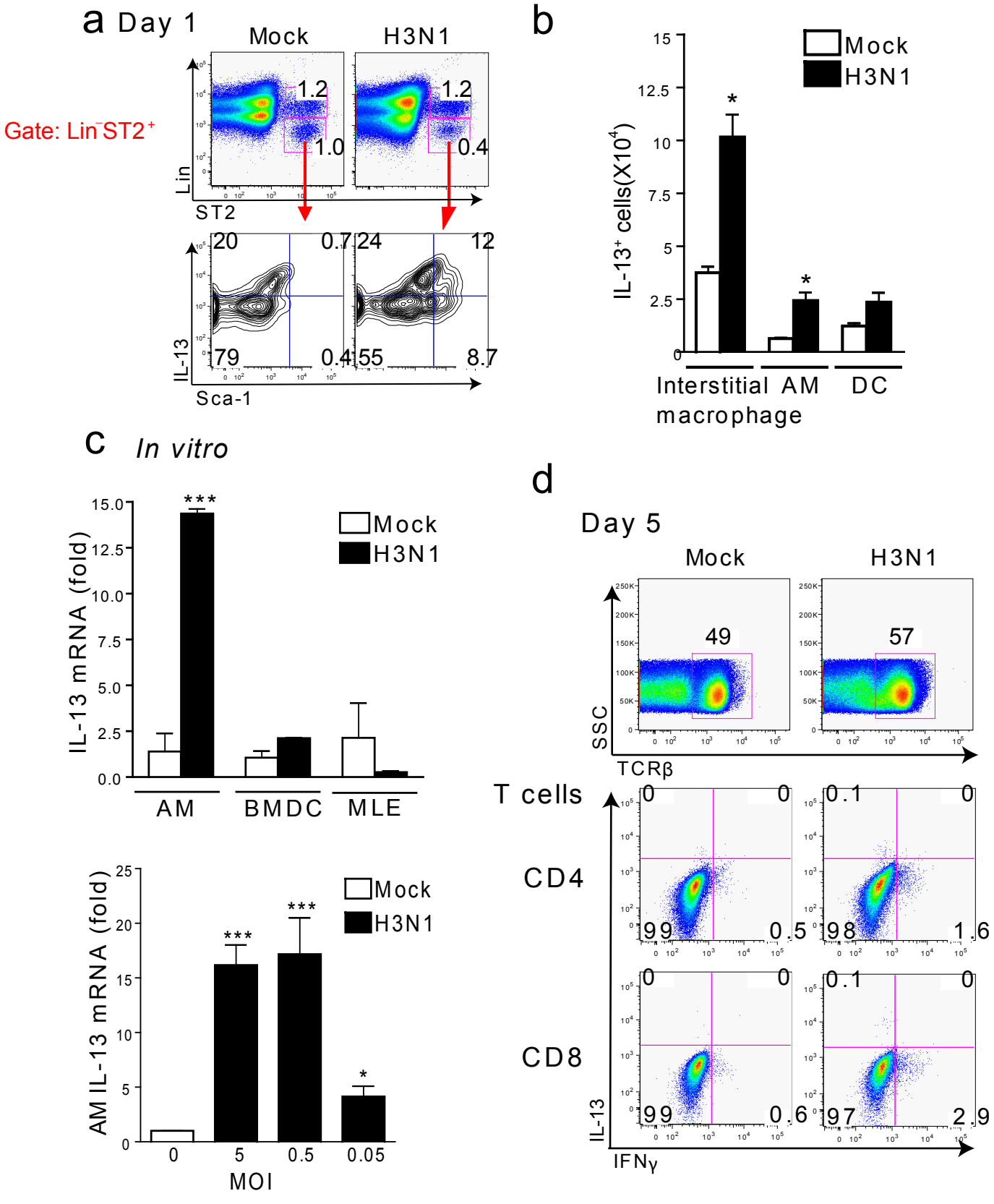

c
***
IL-5 (ng/ml)
Time after infection (d)

Sup Fig 4

Day 5



Sup Fig 5



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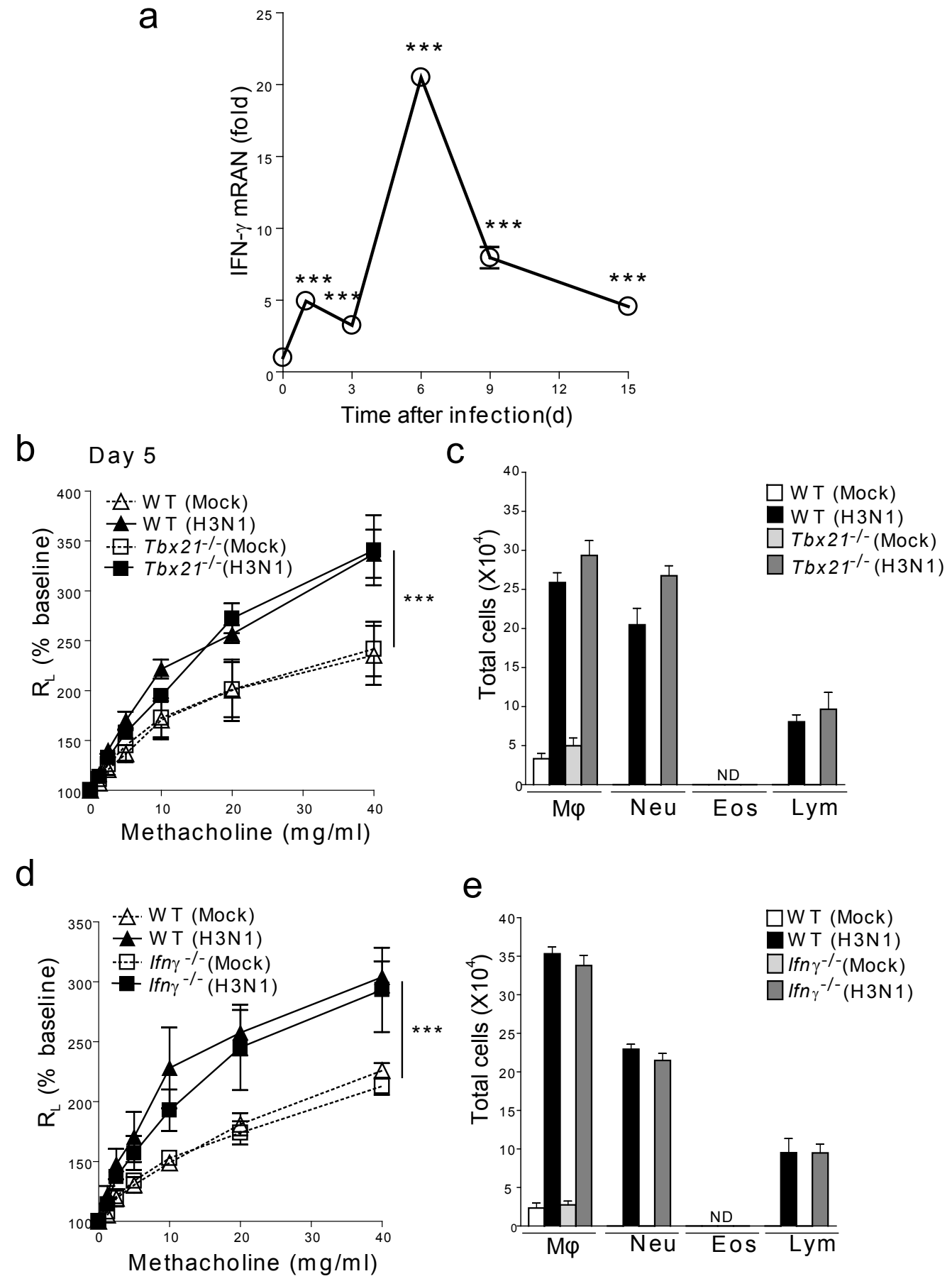
Sup Fig 6



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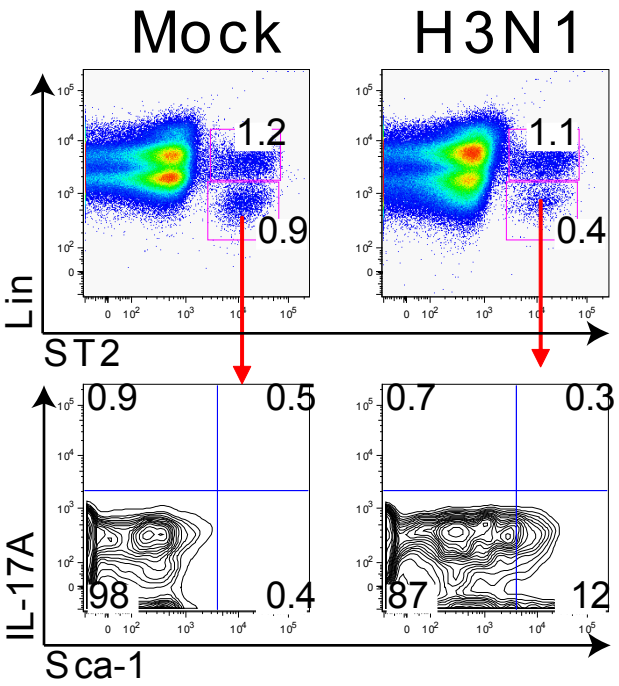
Sup Fig 7

Day 1



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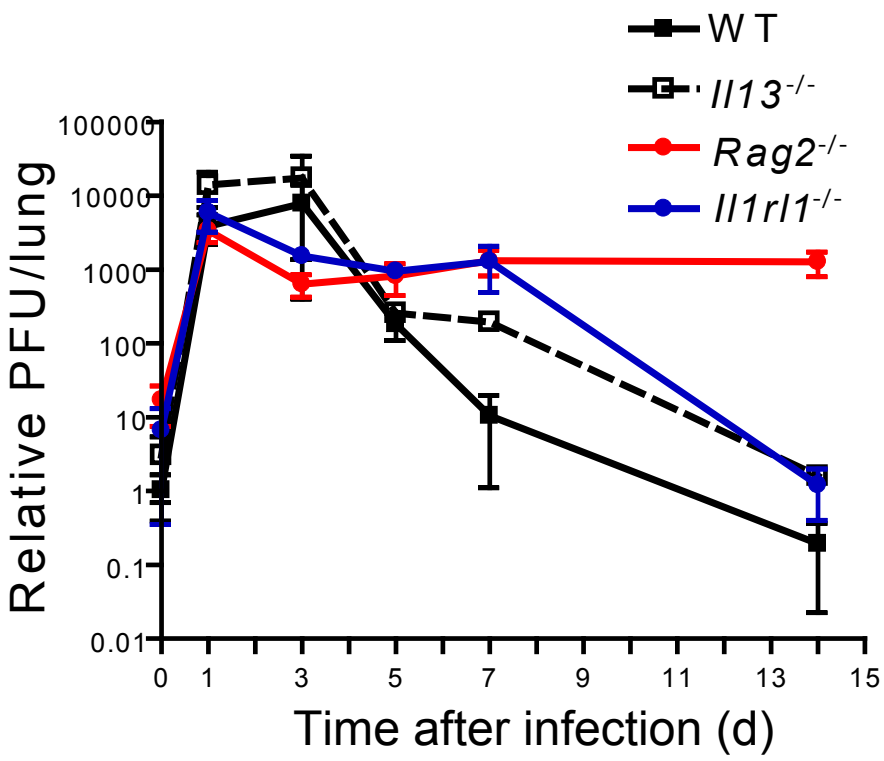
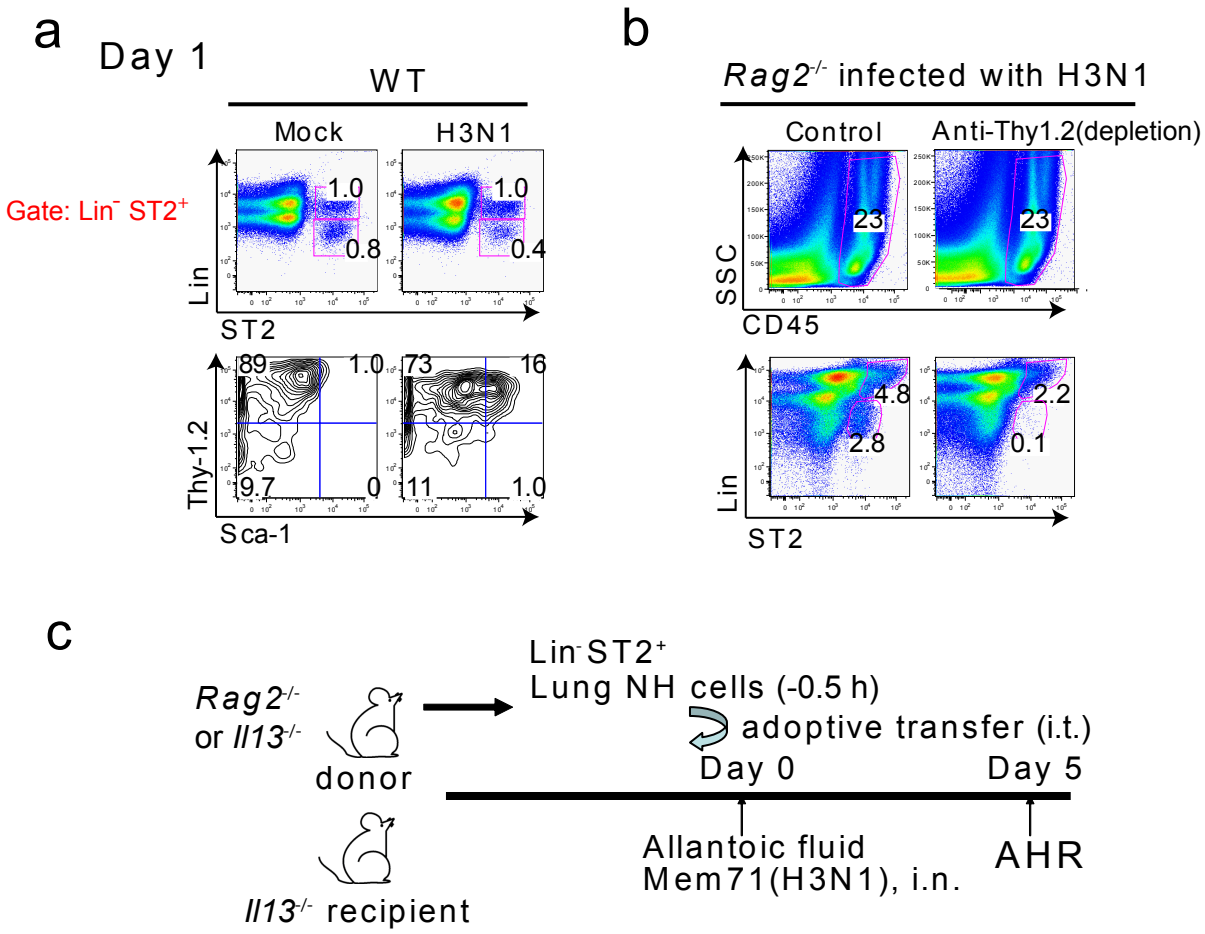
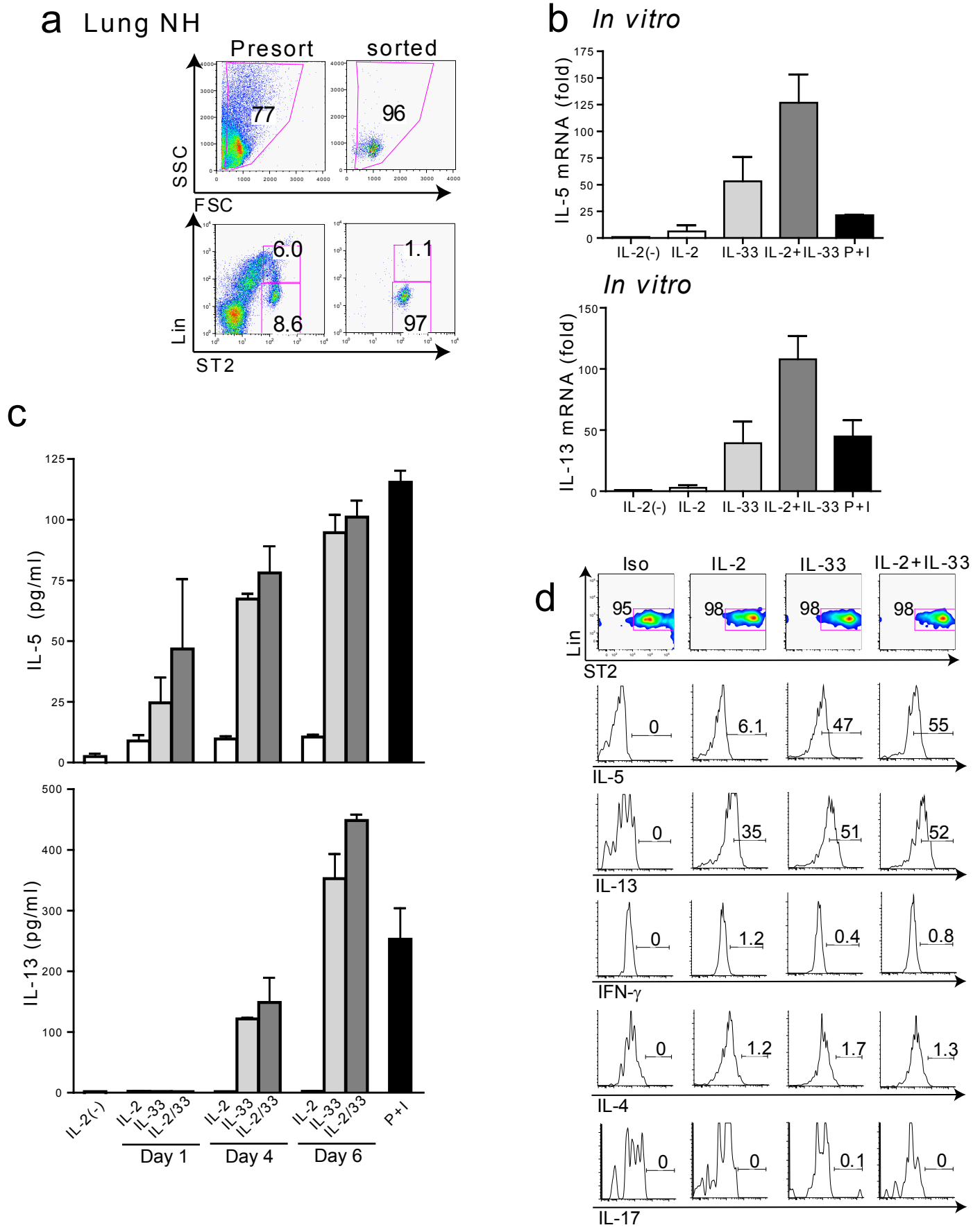
Sup Fig 8


WT
Il13-/-
Rag2-/-
Il1rl1-/-
Relative PFU/lung
100000
10000
1000
100
10
1
0.1
0.01
0
1
3
5
7
9
11
13
15
Time after infection (d)

Sup Fig 9


a
Day 1
WT
Mock
H3N1
Gate: Lin⁻ ST2⁺
1.0
0.8
1.0
0.4
Lin
ST2
89
1.0
9.7
0
73
16
11
1.0
Thy-1.2
Sca-1
b
Rag2-/- infected with H3N1
Control
Anti-Thy1.2(depletion)
23
23
SSC
CD45
4.8
2.8
2.2
0.1
Lin
ST2
c
Rag2-/-
or Il13-/-
donor
Il13-/- recipient
Lin⁻ST2⁺
Lung NH cells (-0.5 h)
adoptive transfer (i.t.)
Day 0
Day 5
Allantoic fluid
Mem71(H3N1), i.n.
AHR

Sup Fig 10



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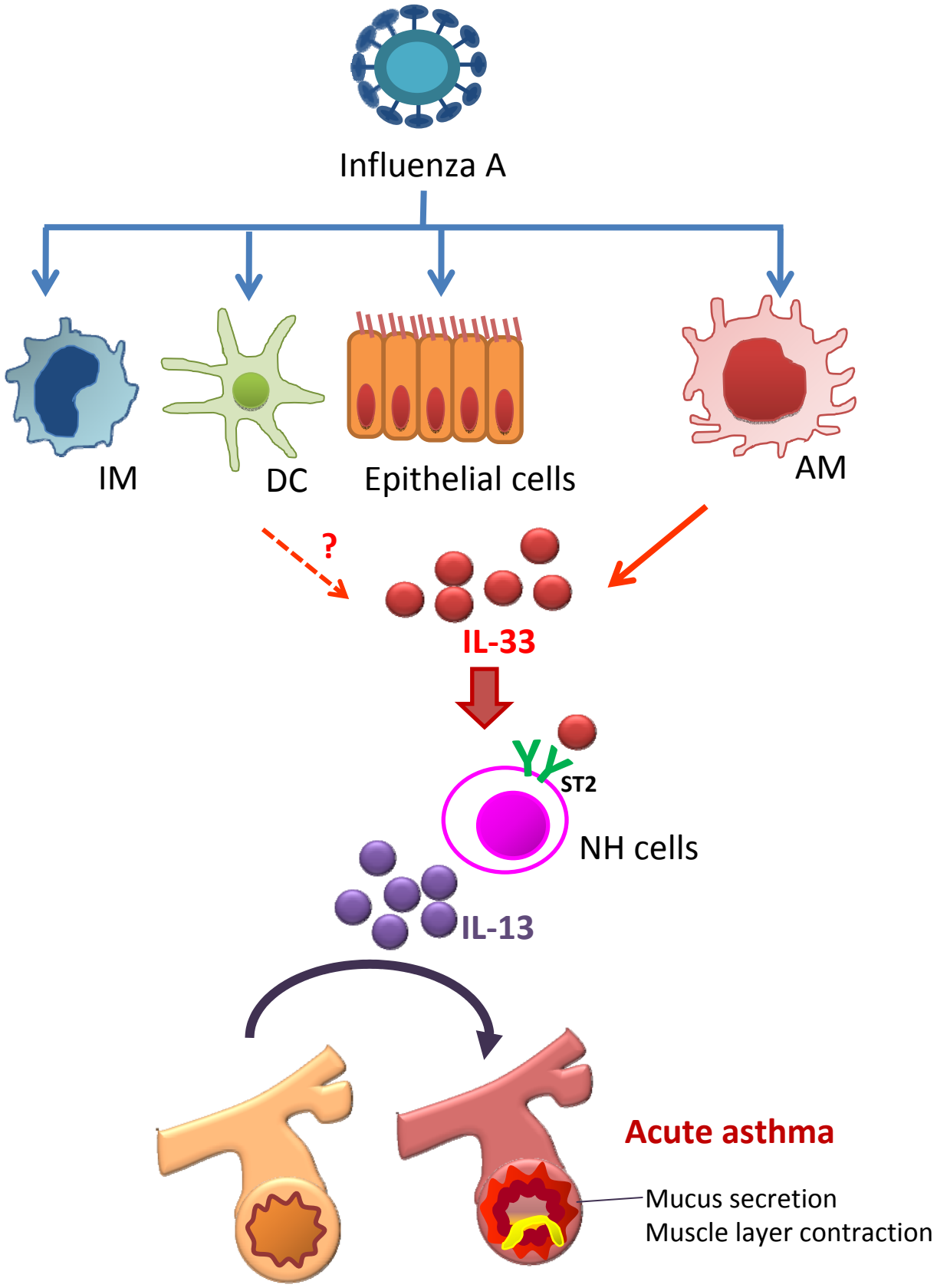
Sup Fig 11



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